METHODS AND COMPOSITIONS FOR MODULATING GLUTAMATE TRANSPORT ACTIVITY IN THE NERVOUS SYSTEM

Related Applications

This application claims priority under 35 U.S.C. §119 to U.S. 60/446,062, filed February 7, 2003, the entire contents of which is hereby incorporated by reference.

Field of the Invention

The invention relates to methods and products for modulating glutamate transport activities in the nervous system. The invention is useful for preventing and treating acute and chronic neurological disorders and injuries that are associated with glutamate excitotoxicity.

Background of the Invention

A devastating feature of a number of neurological disorders and injuries is neuronal cell death. In the 1980s various laboratories identified excess extracellular glutamate, which results in glutamate excitotoxicity, as a factor in the neuronal cell death associated with various neurological disorders and traumas. The mechanism involved in glutamate excitotoxicity has been explored and it is now understood during synaptic activity in normal brain cells, astrocytes play a central role in the uptake of excess glutamate. Astrocytes express high affinity glutamate transporters that remove excess glutamate from the extracellular space, which maintains the concentration of extracellular glutamate at a level below the excitotoxic threshold, therefore preventing glutamate excitotoxicity and resulting neuronal cell death.

The availability of transporter-specific reagents has stimulated studies of transporter expression in animal and human models of disease. Several of these studies indicate that the glutamate transporters are differentially regulated in various pathological conditions. Malfunctioning of glutamate transporter can produce lesions of different severity. In addition, antisense knockdown of glutamate transporters *in vivo* reveals that they play a role in excitotoxicity and prevention of epileptic seizure. Although glutamate transporters have been suspected to play a role in neurophysiology of neurodegenerative diseases with excitotoxic components (e.g. amyotrophic lateral sclerosis [ALS], Alzheimer's disease,

epilepsy, etc), there is a lack of methods available to effectively alter glutamate transport activity and thus prevent and/or treat neurological disorders and disease.

Summary of the Invention

We have developed an assay to determine the ability of compounds to modulate glutamate transport activity. The assay is useful to determine whether a compound has a modulatory effect, and if so, whether the effect is an enhancing or inhibitory effect. The assays can also be used to quantitate the efficacy of a compound in its ability to modulate glutamate transport activity; thus the assay can be used to identify and optimize compounds that are useful to modulate glutamate transport activity. Using this assay, we have identified cloxyquin, citrinin, iodoquinol, oxyquinoline, nordihydroguaiaretic acid (NDGA), ebselen, flunisolide, and hydrocortizone as compounds that enhance glutamate transport. Cloxyquin, citrinin, iodoquinol, and oxyquinoline have not been used in methods to treat glutamate transport-associated disorders and the invention includes the use of these compounds to enhance glutamate transport activity and in treatment of glutamate transport-associated disorders.

Thus, the invention includes methods for discovery of compounds that modulate glutamate transport (e.g. in cells/tissues), methods for treating disorders resulting from or including glutamate excitotoxicity, such as neurological disorders, and compositions for treating such disorders.

According to one aspect of the invention, methods for preventing or treating a glutamate excitoxicity-associated neurological disorder in a subject are provided. The methods include administering to a subject in need of such treatment and otherwise free of indications for treatment with an antimicrobial compound, an effective amount of the antimicrobial compound to treat the neurological disorder. In some embodiments, the antimicrobial compound is cloxyquin or an analog, derivative, or variant thereof that increases glutamate transport activity. In certain embodiments, the antimicrobial compound is citrinin or an analog, derivative, or variant thereof that increases glutamate transport activity. In some embodiments, the antimicrobial compound is iodoquinol or an analog, derivative, or variant thereof that increases glutamate transport activity. In certain embodiments, the antimicrobial compound is oxyquinoline or an analog, derivative, or variant thereof that increases glutamate transport activity. In some embodiments, the glutamate excitotoxicity-associated neurological disorder is selected from the group

consisting of cerebrovascular accident, stroke, seizure, head and spinal cord trauma, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, Huntington's disease, epilepsy, glaucoma, and hepatic encephalopathy. In certain embodiments, the subject is human. In some embodiments, the compound is linked to a targeting molecule. In certain embodiments, the targeting molecule's target is a cell selected from the group consisting of glial cells and neuronal cells. In some embodiments, the compound is a pro-drug. In certain embodiments, the compound is administered prophylactically to a subject at risk of having a glutamate excitotoxicity-associated neurological disorder. In some embodiments, the mode of administration is selected from the group consisting of: implantation, mucosal, injection, inhalation, and oral. In certain embodiments, the compound is administered in combination with an additional drug for treating a neurological disorder. In some embodiments, the methods also include administering a compound selected from the group consisting of nordihydroguaiaretic acid, ebselen, flunisolide, hydrocortisone, and analogs, derivatives, and variants thereof, for prevention and/or treatment of a neurological disorder.

According to another aspect of the invention, methods for treating a subject having a condition characterized by glutamate excitotoxicity are provided. The methods include administering to a subject in need of such treatment a cloxyquin, citrinin, iodoquinol, or oxyquinoline compound, in an amount effective to increase glutamate transport activity, wherein the subject is free of symptoms otherwise calling for treatment with the cloxyquin, citrinin, iodoquinol, and oxyquinoline. In some embodiments, the condition characterized by glutamate excitotoxicity is selected from the group consisting of cerebrovascular accident, stroke, seizure, head and spinal cord trauma, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, epilepsy, glaucoma, and hepatic encephalopathy. In some embodiments, the subject is human. In some embodiments, the cloxyquin, citrinin, iodoquinol, or oxyquinoline compound is linked to a targeting molecule. In certain embodiments, the targeting molecule's target is a cell selected from the group consisting of glial cells and neuronal cells. In some embodiments, the compound is a pro-drug. In certain embodiments, the compound is administered prophylactically to a subject at risk of having the condition characterized by glutamate excitotoxicity. In some embodiments, the mode of administration is selected from the group consisting of: implantation, mucosal, injection, inhalation, and oral. In some embodiments, the compound is administered in combination with an additional drug for treating a neurological disorder.

In some embodiments, the methods also include administering a compound selected from the group consisting of nordihydroguaiaretic acid, ebselen, flunisolide, hydrocortisone, and analogs, derivatives, and variants thereof.

According to one aspect of the invention, methods of evaluating the effect of candidate pharmacological agents on glutamate transport activity are provided. The methods include contacting a test cell sample with a candidate pharmacological agent; contacting the test cell sample with glutamate, determining the effect of the candidate pharmacological agent on the activity of glutamate transport in the cell sample relative to the activity of glutamate transport in a control neuronal cell sample contacted with glutamate and not contacted with the candidate pharmacological agent, wherein a relative increase or relative decrease in the activity of glutamate transport in the test cell sample indicates modulation of glutamate transport activity by the candidate pharmacological agent. In some embodiments, the test cell sample comprises embryonic mouse spinal cord motor neuron hybrid cells (MN-1). In certain embodiments, the glutamate is detectably labeled. In some embodiments, the amount of glutamate transport activity is determined by measuring the amount of detectably labeled glutamate taken up by the test cell sample. In certain embodiments, a relative increase in the activity of glutamate transport in the test cell sample indicates the modulator is a glutamate transport enhancing agent. In certain embodiments, a relative decrease in the activity of glutamate transport in the test cell sample indicates the modulator is a glutamate transport inhibitory agent.

According to another aspect of the invention, methods for preparing an animal model of a disorder characterized by glutamate excitotoxicity are provided. The methods include introducing a glutamate transport inhibitory agent into a non-human animal. In some embodiments, the methods also include detecting in the non-human animal symptoms of a disorder characterized by glutamate excitotoxicity. In certain embodiments, the animal model is a model for a neurological disorder. In some embodiments, the disorder selected from the group consisting of: cerebrovascular accident, stroke, seizure, head and spinal cord trauma, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, epilepsy, glaucoma, and hepatic encephalopathy.

According to another aspect of the invention, kits are provided. The kits include a package housing a first container containing an antimicrobial compound, and instructions for using the antimicrobial compound in the prevention and/or treatment of a neurological disorder. In some embodiments, the antimicrobial compound is selected from the group

consisting of: cloxyquin, citrinin, iodoquinol, oxyquinoline, and analogs, derivatives, and variants thereof. In certain embodiments, the kits also include a second container containing a medication and instruction for the using the compounds and medication for prevention and/or treatment of a neurological disorder. In certain embodiments, the kits also include a container containing a compound selected from the group consisting of nordihydroguaiaretic acid, ebselen, flunisolide, hydrocortisone, and analogs, derivatives, and variants thereof; and instructions for using the compounds for prevention and/or treatment of a neurological disorder. In some embodiments, the antimicrobial compound is formulated for delivery to neuronal cells. In certain embodiments, the antimicrobial compound is formulated for delivery to glial cells. In some embodiments, the antimicrobial compound is formulated for sustained release.

According to yet another aspect of the invention, methods of modulating glutamate transport activity in a subject in need of such treatment are provided. The methods include administering a compound selected from the group consisting of nordihydroguaiaretic acid (NDGA), ebselen, flunisolide, hydrocortisone, and analogs, derivatives, and variants thereof, in an amount effective for modulating glutamate transport activity. In some embodiments, the subject is human. In certain embodiments, the compound is linked to a targeting molecule. In some embodiments, the targeting molecule's target is a cell selected from the group consisting of glial cells and neuronal cells. In certain embodiments, the compound is a pro-drug. In some embodiments, the compound is administered prophylactically to a subject at risk of having a glutamate excitotoxicity-associated neurological disorder. In certain embodiments, the glutamate excitotoxicity-associated neurological disorder is selected from the group consisting of cerebrovascular accident, stroke, seizure, head and spinal cord trauma, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, epilepsy, glaucoma, and hepatic encephalopathy. In some embodiments, the mode of administration is selected from the group consisting of: implantation, mucosal, injection, inhalation, and oral. In some embodiments, the compound is administered in combination with an additional drug for treating a neurological disorder. In certain embodiments, modulating glutamate transport activity is increasing glutamate transport activity.

According to one aspect of the invention, methods of decreasing glutamate excitotoxicity in a subject in need of such treatment are provided. The methods include administering a compound selected from the group consisting of nordihydroguaiaretic acid (NDGA), ebselen, flunisolide, hydrocortisone and analogs, derivatives, and variants thereof,

an amount effective for decreasing glutamate excitotoxicity in the subject. In some embodiments, the glutamate excitotoxicity is associated with a neurological disorder selected from the group consisting of cerebrovascular accident, stroke, seizure, head and spinal cord trauma, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, epilepsy, glaucoma, and hepatic encephalopathy. In certain embodiments, the subject is human. In some embodiments, the compound is linked to a targeting molecule. In some embodiments, the targeting molecule's target is a cell selected from the group consisting of glial cells and neuronal cells. In some embodiments, the compound is a pro-drug. In some embodiments, the compound is administered prophylactically to a subject at risk of having a glutamate excitotoxicity-associated neurological disorder. In certain embodiments, the mode of administration is selected from the group consisting of: implantation, mucosal, injection, inhalation, and oral. In some embodiments, the compound is administered in combination with an additional drug for treating a neurological disorder.

According to one aspect of the invention, compositions are provided. The compositions include an antimicrobial compound and a compound for preventing and/or treating a neurological disorder. In some embodiments, the antimicrobial compound is selected from the group consisting of cloxyquin, citrinin, iodoquinol, oxyquinoline, and analogs, derivatives, and variants thereof. In certain embodiments, the compound for treating a neurological disorder is selected from the group consisting of: nordihydroguaiaretic acid (NDGA), ebselen, flunisolide, hydrocortisone, and analogs, derivatives, and variants thereof. In some embodiments, the neurological disorder is selected from the group consisting of cerebrovascular accident, stroke, seizure, head and spinal cord trauma, amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, epilepsy, glaucoma, and hepatic encephalopathy. In some embodiments, the compound is linked to a targeting molecule. In certain embodiments, the targeting molecule's target is a cell selected from the group consisting of glial cells and neuronal cells. In some embodiments, the compound is a pro-drug.

Use of the glutamate-transport modulating compositions in the manufacture of a medicament for treating glutamate uptake disorders including glutamate uptake disorders and neurological disorders, also is provided.

Brief Description of the Drawings

Figure 1 shows a photomicrographic image of embryonic mouse spinal cord motor neuron hybrid cells (MN-1) in culture (Fig. 1A). The MN-1 cells express GLT1, GLAST and EAAC1 glutamate transporters. Fig. 1B shows a digitized image of a Western blot demonstrating Western analysis of MN-1 total homogenates (PBS-SDS 0.1% + protease inhibitors). The Western blot was probed with antibodies against glutamate transporters at two different concentrations and visualized by ECL.

Figure 2 shows graphs of glutamate uptake. Fig. 2A shows Michaelis-Menten analysis of glutamate uptake in MN-1 cells. Uptake was linear for up to 30 min and displayed high-affinity and high-capacity for glutamate (Km ~ 30 μM; Vmax ~ 200 pmol/min/mg protein). Fig. 2B shows dihydrokainate (DHK) -sensitivity of glutamate uptake. Different concentrations of DHK were applied in presence of 0.5 μM glutamate (1:2,000; isotopically diluted with ^{3}H-L-glutamate). The uptake was measured for 15 minutes before stopping the reaction with cold choline buffer. About 40% of the total glutamate uptake exhibited by MN-1 cells is sensitive to dihydrokainate, a potent and irreversible inhibitor of GLT1, with IC_{50} of ≈160 μM.

Figure 3 is a graph demonstrating the effect on glutamate uptake of compounds from microplate #13 of the NINDS custom collection. One positive hit was selected from the screening of this microplate. We set an increase of 30% as cut-off for compounds to be considered positive. Such increase is approximately 4-5 times the standard deviation of the value of glutamate uptake in control non-treated MN-1 cells.

Figure 4 shows the chemical structures of the positive hits in the screening assay. Compounds have been grouped in two different categories (Fig. 4A and Fig. 4B, respectively) according to their chemical analogies and mechanisms of action.

Figure 5 shows histograms of glutamate uptake measured in forebrain synaptosomes from NDGA and placebo-treated mice. Fig. 5A shows the effect of NDGA (10mg/10 days) on glutamate uptake measured in forebrain synaptosomes of control mice. Fig. 5B shows glutamate uptake in synaptosomes in control mice (single values/mouse).

Figure 6 is a schematic diagram of the NDGA acute treatment regimen in the SOD1-G93A mouse model of ALS as a test of the therapeutic effect of NDGA in ALS. The tests included 10 days of treatment with NDGA in 100 day old SOD1-G93A mice.

Figure 7 is a graph of the survival curve illustrating the survival time of NDGA-treated and placebo-treated SOD1-G93A mice.

Detailed Description of the Invention

The methods of the invention involve the administration of compounds that modulate the glutamate transport activity in neuronal tissues. As used herein the term "modulate" means enhance or inhibit. Compositions of the invention include compounds that modulate the activity of glutamate transport mechanisms in cells, tissues, and subjects. The methods of the invention involve the administration of compounds that modulate glutamate transport activity in neuronal cells and/or tissues and therefore are useful to reduce or prevent the damage from glutamate excitotoxic activity that may occur in acute and chronic neurological disorders. As used herein, the term "glutamate excitotoxicity-associated disorder" is used interchangeably with the term "neurological disorder" and includes, but is not limited to: cerebrovascular accidents (stroke), seizure, head and spinal cord trauma, and neurodegenerative disorders, including, but not limited to: amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, epilepsy, hepatic encephalopathy, glaucoma, and Huntington's disease. We have discovered that the deleterious effects seen in these disorders that are triggered by excitotoxicity can be ameliorated by the administration of the compositions of the invention. The compositions of the invention include compounds that enhance glutamate transport activity (glutamate uptake) and/or inhibit excitotoxicity in cells and tissues of subjects, thereby reducing the excitotoxic damage that is associated with the neurological disorders.

As used herein, the term "subject" means any mammal that may be in need of treatment with the glutamate transport modulating compounds of the invention. Subjects include but are not limited to: humans, non-human primates, cats, dogs, sheep, pigs, horses, cows, rodents such as mice, rats, etc. Table 1 provides glutamate transport enhancing compounds of the invention and includes: cloxyquin, citrinin, iodoquinol, and oxyquinoline.

Table 1. Glutamate transport enhancing compounds.

Primary Name	Additional names	Alternative function
Cloxyquin	5-chloro-8-quinolinol; 5-chlorohydroxyquinoline, 5-chloro-8-oxychinolin, cloxiquine	Antibacterial
Citrinin	(3R-trans)-4,6-dihydro-8-hydroxy 3,4,5-trimethyl-6-oxo-3H-2-benzypyran-7-carboxylic acid. Antimycin	Antibiotic
Iodoquinol	5,7-Diiodo-8-quinolinol; diiodohydrooxyquin; diiodo-oxyquinoline; 5,7-diiodo-8-hydroxyquinoline; SS 578; Diodoquin; Di-Quinol; Disoquin; Floraquin; Dyodin; Dinoleine; Searlequin; Diodoxylin; Moebiquin; Rafamebin; Ioquin; Direxiode; Stanquinate; Quinadome, Yodoxin; Qoaquin; Enterosept, Embequin	Antiamebic
Oxyquinoline		Antiseptic
Nordihydroguaiaretic acid (NDGA)		Lipoxygenase inhibitor Antioxidant, Anti-inflammatory, Antineoplastic
Ebselen		Neuroprotective, antioxidant, Lipoxygenases inhibitor
Flunisolide		Corticosteriod, Anti-inflammatory, antiasthma
Hydrocortisone	Hydrocortizone	Corticosterioid, Anti-inflammatory

The antimicrobial compounds, cloxyquin, citrinin, iodoquinol, and oxyquinoline are known to act as antibacterial, antibiotic, antiamebic, and antiseptic agents respectively. The foregoing compounds have never before been given to patients to enhance glutamate transport activity and/or to treat or prevent glutamate excitotoxic damage in subjects who are otherwise free of indications for their administration. Preferably, the cloxyquin, citrinin, iodoquinol, and oxyquinoline compounds of the invention are administered to subjects that are free of indications for antibacterial, antibiotic, antiamebic, and antiseptic treatment, respectively. By "free of indications for treatment with an antibacterial, antibiotic, antiamebic, or antiseptic," it is meant that the subject does not have symptoms that call for treatment with an antibacterial, antibiotic, antiamebic or antiseptic (other than the indication that exists as a result of this invention). As used herein, the term "antimicrobial compound" means a compound that is an antibacterial, antibiotic, antiamebic or antiseptic compound. Thus, antimicrobial compounds of the invention include cloxyquin, citrinin, iodoquinol, and oxyquinoline, which are antibacterial, antibiotic, antiamebic and antiseptic compounds, respectively.

The methods of the invention include administration of glutamate transport enhancing compounds that preferentially target neuronal cells and/or tissues. In addition, the compounds can be specifically targeted to neuronal tissue (e.g. glial cells and/or neuronal

cells) using various delivery methods, including, but not limited to: administration to neuronal tissue, the addition of targeting molecules to direct the compounds of the invention to neuronal tissues (e.g. glial cells and/or neuronal cells), etc. The addition of a targeting molecule can be done through covalent or non-covalent attachment (linking) of a compound of the invention to a molecule that will target a tissue or cell type of interest. Additional methods to specifically target molecules and compositions of the invention to brain tissue and/or neuronal tissues are known to those of ordinary skill in the art.

The invention involves, in part, the administration of a compound that enhances glutamate transport activity in the nervous system. As used herein, the term "glutamate transport activity" means the uptake of extracellular glutamate. After glutamate is released from a synapse, it is necessary to have the excess extracellular glutamate removed to prevent the postsynaptic neuron from being excited indefinitely. Uptake of extracellular glutamate is known to take place via glutamate transporters, examples of which, although not intended to be limiting include: EAAT1, GLT1 (also known as EAAT2), and GLAST. Glutamate transporters are expressed by glial cells and neurons and these high affinity glutamate transporters help maintain extracellular glutamate concentration below the levels at which excitotoxicity and/or neuronal cell death will occur.

As used herein, the term "enhance" means to increase the level of glutamate transport activity to a level or amount that is statistically significantly more than a control level of glutamate transport activity. In some cases, the increase in the level of glutamate transport activity means the level of glutamate transport activity is raised from zero to a level above zero, in other cases an increase in glutamate transport activity means an increase from a level that is above zero to a level significantly higher than that original or baseline level of activity. A control level of glutamate transport activity is the level of activity that represents the normal level of glutamate transport activity in a subject. In some instances, a control level will be the level in a disorder-free subject, in other instances a control level will be the level in a subject who has a neurological disorder or brain trauma. These types of control levels are useful in assays to assess the efficacy of a glutamate transport modulating compound of the invention.

It will be understood by one of ordinary skill in the art that a control level of glutamate transport activity may be a predetermined value, which can take a variety of forms. It can be a single value, such as a median or mean. It can be established based upon comparative groups, such as in disease-free groups that have normal levels of glutamate

transport activity. Other comparative groups may be groups of subjects with specific neurological disorders, e.g. ALS, Parkinson's disease, Huntington's disease, or Alzheimer's disease, hepatic encephalopathy, epilepsy, glaucoma or may be a group of subjects with a specific nature or type of brain trauma.

In some embodiments, a compound that enhances glutamate transport activity is an agent that reduces glutamate excitotoxicity. The level of excitotoxicity may be one that is below the level seen in subjects with a neurological disorder

The invention relates in part to the administration of a glutamate transport enhancing compound of the invention in an amount effective to treat or prevent excitotoxic damage in nervous system tissues in subjects with neurological disorders or brain trauma.

In some aspects of the invention, the glutamate transport modulating compounds include functional analogs, derivatives, and variants of the glutamate transport modulating compounds. For example, functional analogs, derivatives, and variants of the glutamate transport enhancing compounds of Table 1 can be made, for example, to enhance a property of a compound, such as stability. Functional analogs, derivatives, and variants of the compounds of Table 1 may also be made to provide a novel activity or property to a compound of Table 1, for example, to enhance detection. In some embodiments of the invention, modifications to a glutamate transport enhancing molecule of the invention, can be made to the structure or side groups of the compound and can include deletions, substitutions, and additions of atoms, or side groups. Alternatively, modifications can be made by addition of a linker molecule, addition of a detectable moiety, such as biotin or a fluorophore, chromophore, enzymatic, and/or radioactive label, and the like.

Analogs of the cloxyquin, citrinin, iodoquinol and oxyquinoline molecules that retain some or all of the glutamate transport enhancing activity of the cloxyquin, citrinin, iodoquinol and oxyquinoline molecules, respectively, also can be used in accordance with the invention. In some embodiments, an analog of a molecule may have a higher level of glutamate transport activity than that molecule. Substitutions may include one or more chemical groups of the molecules. Chemical groups that can be added to or substituted in the molecules include: hydrido, alkyl, alkenyl, alkynyl, cycloalkyl, heterocycyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, acyl, amino, acyloxy, acylamino, carboalkoxy, carboxyamido, carboxyamido, halo and thio groups. Substitutions can replace one or more chemical groups or atoms on the molecules.

Molecular terms, when used in this application, have their common meaning unless otherwise specified. The term "hydrido" denotes a single hydrogen atom (H). The term "acyl" is defined as a carbonyl radical attached to an alkyl, alkenyl, alkynyl, cycloalkyl, heterocycyl, aryl or heteroaryl group, examples of such radicals being acetyl and benzoyl. The term "amino" denotes a nitrogen radical containing two substituents independently selected from the group consisting of hydrido, alkyl, cycloalkyl, heterocyclyl, aryl, and heteroaryl. The term "acyloxy" denotes an oxygen radical adjacent to an acyl group. The term "acylamino" denotes a nitrogen radical adjacent to an acyl group. The term "carboalkoxy" is defined as a carbonyl radical adjacent to an alkoxy or aryloxy group. The term "carboxyamido" denotes a carbonyl radical adjacent to an amino group. The term "carboxy" embraces a carbonyl radical adjacent to an alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl or heteroaryl group. The term "halo" is defined as a bromo, chloro, fluoro or iodo radical. The term "thio" denotes a radical containing a substituent group independently selected from hydrido, alkyl, cycloalkyl, heterocyclyl, aryl and heteroaryl, attached to a divalent sulfur atom, such as, methylthio and phenylthio.

The term "alkyl" is defined as a linear or branched, saturated radical having one to about ten carbon atoms unless otherwise specified. Preferred alkyl radicals are "lower alkyl" radicals having one to about five carbon atoms. One or more hydrogen atoms can also be replaced by a substitutent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of alkyl groups include methyl, *tert*-butyl, isopropyl, and methoxymethyl. The term "alkenyl" embraces linear or branched radicals having two to about twenty carbon atoms, preferably three to about ten carbon atoms, and containing at least one carbon-carbon double bond. One or more hydrogen atoms can also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of alkenyl groups include ethylenyl or phenyl ethylenyl. The term "alkynyl" denotes linear or branched radicals having from two to about ten carbon atoms, and containing at least one carbon-carbon triple bond. One or more hydrogen atoms can also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl.

Examples of alkynyl groups include propynyl. The term "aryl" denotes aromatic radicals in a single or fused carbocyclic ring system, having from five to twelve ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of aryl groups include phenyl, naphthyl, biphenyl, and terphenyl. "Heteroaryl" embraces aromatic radicals which contain one to four hetero atoms selected from oxygen, nitrogen and sulfur in a single or fused heterocyclic ring system, having from five to fifteen ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of heteroaryl groups include, pyridinyl, thiazolyl, thiadiazoyl, isoquinolinyl, pyrazolyl, oxazolyl, oxadiazoyl, triazolyl, and pyrrolyl groups.

The term "cycloalkyl" is defined as a saturated or partially unsaturated carbocyclic ring in a single or fused carbocyclic ring system having from three to twelve ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of a cycloalkyl group include cyclopropyl, cyclobutyl, cyclohexyl, and cycloheptyl. The term "heterocyclyl" embraces a saturated or partially unsaturated ring containing zero to four hetero atoms selected from oxygen, nitrogen and sulfur in a single or fused heterocyclic ring system having from three to twelve ring members. One or more hydrogen atoms may also be replaced by a substituent group selected from acyl, amino, acylamino, acyloxy, carboalkoxy, carboxy, carboxyamido, cyano, halo, hydroxy, nitro, thio, alkyl, alkenyl, alkynyl, cycloalkyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, sulfoxy, and formyl. Examples of a heterocyclyl group include morpholinyl, piperidinyl, and pyrrolidinyl. The term "alkoxy" denotes oxy-containing radicals substituted with an alkyl, cycloalkyl or heterocyclyl group. Examples include methoxy, *tert*-butoxy, benzyloxy and cyclohexyloxy. The term "aryloxy" denotes oxy-containing radicals substituted with an aryl or heteroaryl group. Examples include phenoxy. The term "sulfoxy" is defined as a hexavalent sulfur radical bound to two or three substituents selected from the group

consisting of oxo, alkyl, cycloalkyl, heterocyclyl, aryl and heteroaryl, wherein at least one of said substituents is oxo.

The glutamate transport modulating compounds of the invention also include, but are not limited to any pharmaceutically acceptable salts, esters, or salts of an ester of the compound. Examples of salts that may be used, which is not intended to be limiting include: chloride, acetate, hydrochloride, methansulfonate or other salt of a compound of Table 1 or a functional analog, derivative, variant, or fragment of the compound.

Derivatives of the compounds of Table 1 include compounds which, upon administration to a subject in need of such administration, deliver (directly or indirectly) a pharmaceutically active glutamate transport modulating compound as described herein. An example of pharmaceutically active derivatives of the invention includes, but is not limited to, pro-drugs. A pro-drug is a derivative of a compound that contains an additional moiety that is susceptible to removal in *vivo* yielding the parent molecule as a pharmacologically active agent. An example of a pro-drug is an ester that is cleaved *in vivo* to yield a compound of interest. Pro-drugs of a variety of compounds, and materials and methods for derivatizing the parent compounds to create the pro-drugs, are known to those of ordinary skill in the art and may be adapted to the present invention.

Analogs, variants, and derivatives of the compounds in Table 1 of the invention may be identified using standard methods known to those of ordinary skill in the art. Useful methods involve identification of compounds having similar chemical structure, similar active groups, chemical family relatedness, and other standard characteristics. For the purposes of this invention, the chemical elements are identified in accordance with the Periodic Table of the Elements, CAS version, Handbook of Chemistry and Physics 75th Ed., inside cover, and specific functional groups are defined as described therein. Additionally, general principles of organic chemistry, as well as specific functional moieties and reactivity, are described in "Organic Chemistry", Thomas Sorrell, University Science Books, Sausalito. 1999, the contents of which are incorporated herein by reference in their entirety.

Using the structures of the compounds disclosed herein (see Figure 4), one of ordinary skill in the art is enabled to make predictions of structural and chemical motifs for analogs, variants, and/or derivatives that possess similar functions of the compounds disclosed in Table 1. Using structural motifs as search, evaluation, or design criteria, one of ordinary skill in the art is enabled to identify classes of compounds (functional variants of the glutamate transport enhancing or inhibiting molecules or agents) that possess the modulatory function

of the compounds disclosed herein. These compounds may be synthesized using standard synthetic methods and tested for activity as described herein.

The invention also involves methods for determining the functional activity of glutamate transport modulating compounds described herein. The function or status of a compound as a glutamate transport-modulating compound can be determined according to assays known in the art or described herein. For example, cells can be contacted with a candidate glutamate transport-modulating compound under conditions that produce excitotoxic damage and/or activate glutamate transport activity, and standard procedures can be used to determine whether glutamate transport activity is modulated by the compound and/or whether the excitotoxicity level is modulated by the compound. Such methods may also be utilized to determine the status of analogs, variants, and derivatives as inhibitors of glutamate transport activity and/or excitotoxicity or enhancers of glutamate transport activity and/or excitotoxicity. Although not intended to be limiting, an example of a method with which the ability of a glutamate transport modulating compound to modulate glutamate transport activity can be tested, is an *in vitro* assay system that utilizes detectably labeled glutamate (e.g. L-glutamate) molecules. As used herein, the term "detectably labeled" means labeled with a marker that can be detected using visual or other means. Examples of detectable labels include, but are not limited to, radiolabels, fluorophores, and chromophores.

In the methods of the invention, detectably labeled glutamate can be used to quantitate the number of glutamate molecules transported in a tissue or cell sample. Such an assay is described herein (see Example 1). A first detectable signal may be used to identify the cellular localization of glutamate molecules in the assay system and the level of transport activity can be assessed by monitoring the localization of the glutamate molecules. The transport activity can be measured in the system both before and after contacting the system with a candidate glutamate transport modulating compound as an indication of the effect of the compound on the level of glutamate transport activity. Secondary screens may further be used to verify the compounds identified as enhancers or inhibitors of glutamate transport activity. In addition, analogs of glutamate transport modulating compounds can be tested for their glutamate transport modulating activity by using an activity assay (see example 1). An example of an assay method, although not intended to be limiting, is contacting a tissue or cell sample with a glutamate transport modulating compound and determining the compound's modulatory activity as described herein. Contacting a similar cell or tissue sample with an analog of the glutamate transport modulating compound, determining its

activity, and then comparing the two activity results as a measure of the efficacy of the analog's glutamate transport modulating activity.

In addition to the *in vitro* assays described above, an *in vivo* assay may be used to determine the functional activity of glutamate transport modulating compounds described herein. In such assays, animals exposed to brain injury or animal models of neurological disorders can be treated with a glutamate transport modulating compound of the invention. Glutamate transport activity and/or excitotoxic damage may be assayed by glutamate localization assays using methods such as labeling or imaging methods. In addition, excitotoxic damage may be assayed more directly by histopathologic examination of brains Additionally, animals with and without glutamate transport modulating compound treatment can be examined for behavior and/or survival as an indication of the effectiveness and/or efficacy of the compounds. Behavior may be assessed by examination of symptoms of excitotoxic damage as described herein below. These measurements can then be compared to corresponding measurements in control animals. For example, test and control animals may be examined following administration of a glutamate transport modulator (enhancer or inhibitor) of the invention. In some embodiments, test animals are administered a glutamate transport modulating compound of the invention and control animals are not. Any resulting excitotoxic damage can then be determined for each type of animal using known methods in the art as described herein. Such assays may be used to compare levels of glutamate transport or excitotoxic damage in animals administered the candidate glutamate transport modulating compound to control levels of excitotoxic damage or glutamate transport in animals not administered the glutamate transport modulating compound as an indication that the putative glutamate transport modulating compound is effective to modulate glutamate transport activity and/or excitotoxicity.

Once one or more glutamate transport modulating compounds are verified as modulating glutamate transport activity and/or excitotoxicity using assays as described herein (e.g., in Example 1), further biochemical and molecular techniques may be used to identify the targets of these compounds and to elucidate the specific roles that these target molecules play in the process of glutamate transport and/or excitotoxicity. An example, though not intended to be limiting, is that the compound(s) may be labeled and contacted with a cell to identify the host cell proteins with which these compounds interact. Such proteins may be purified, e.g., by labeling the compound with an immunoaffinity tag and applying the protein-bound compound to an immunoaffinity column.

A glutamate transport modulating compound of the invention may be delivered to the cell using standard methods known to those of ordinary skill in the art. Various techniques may be employed for introducing glutamate transport modulating compounds of the invention to cells, depending on whether the compounds are introduced *in vitro* or *in vivo* in a host. In some embodiments of the invention, a glutamate transport modulating compound of the invention may be delivered in the form of a delivery complex. The delivery complex may deliver the glutamate transport-modulating compound into any cell type, or may be associated with a molecule for targeting a specific cell type. Examples of delivery complexes include a glutamate transport modulating compound of the invention associated with: a sterol (e.g., cholesterol), a lipid (e.g., a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g., an antibody, including but not limited to monoclonal antibodies, or a ligand recognized by target cell specific receptor). Some complexes may be sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex can be cleavable under appropriate conditions within the cell so that the glutamate transport modulating compound is released in a functional form.

An example of a targeting method, although not intended to be limiting, is the use of liposomes to deliver a glutamate transport modulating compound of the invention into a cell. Liposomes may be targeted to a particular tissue, such as glial or neuronal cells, by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Such proteins include proteins or fragments thereof specific for a particular cell type, antibodies for proteins that undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half life, and the like.

For certain uses, it may be desirable to target the compound to particular cells, for example specific neuronal cells, or nervous system cells, e.g., to glial cells. In such instances, a vehicle (e.g. a liposome) used for delivering a glutamate transport modulating compound of the invention to a cell type (e.g. a neuronal or glial cell) may have a targeting molecule attached thereto that is an antibody specific for a surface membrane polypeptide of the cell type or may have attached thereto a ligand for a receptor on the cell type. Such a targeting molecule can be bound to or incorporated within the glutamate transport modulating compound delivery vehicle. Where liposomes are employed to deliver the glutamate transport modulating compounds of the invention, proteins that bind to a surface membrane protein associated with endocytosis may be incorporated into the liposome formulation for targeting and/or to facilitate uptake.

Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN™ and LIPOFECTACE™, which are formed of cationic lipids such as N-[1-(2,3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications. Liposomes also have been reviewed by Gregoriadis, G. in Trends in Biotechnology, V. 3, p. 235-241 (1985).

When administered, the glutamate transport modulating compounds (also referred to herein as therapeutic compounds and/or pharmaceutical compounds) of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents.

The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredients. The characteristics of the carrier will depend on the route of administration.

The therapeutics of the invention can be administered by any conventional route, including injection or by gradual infusion over time. The administration may, for example, be oral, intravenous, intraperitoneal, intramuscular, intranasal, intracavity, subcutaneous, intradermal, or transdermal.

The therapeutic compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the compounds into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the therapeutic agent into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the therapeutic agent, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using those suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1, 3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland

fixed oil may be employed including synthetic mono or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Carrier formulations suitable for oral, subcutaneous, intravenous, intramuscular, etc. can be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA.

Compositions suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets, or lozenges, each containing a predetermined amount of the therapeutic agent. Other compositions include suspensions in aqueous liquors or non-aqueous liquids such as a syrup, an elixir, or an emulsion.

The invention provides a composition of the above-described agents for use as a medicament, methods for preparing the medicament and methods for the sustained release of the medicament *in vivo*. Delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the therapeutic agent of the invention, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer-based systems such as polylactic and polyglycolic acid, poly(lactide-glycolide), copolyoxalates, polyanhydrides, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polycaprolactone. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Pat. No. 5,075,109. Nonpolymer systems that are lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono-, di- and tri-glycerides; phospholipids; hydrogel release systems; silastic systems; peptide based systems; wax coatings, compressed tablets using conventional binders and excipients, partially fused implants and the like. Specific examples include, but are not limited to: (a) erosional systems in which the polysaccharide is contained in a form within a matrix, found in U.S. Patent Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

In one particular embodiment, the preferred vehicle is a biocompatible microparticle or implant that is suitable for implantation into the mammalian recipient. Exemplary bioerodible implants that are useful in accordance with this method are described in PCT International application no. PCT/US/03307 (Publication No. WO 95/24929, entitled "Polymeric Gene Delivery System". PCT/US/03307 describes a biocompatible, preferably

biodegradable polymeric matrix for containing an exogenous gene under the control of an appropriate promoter. The polymeric matrix is used to achieve sustained release of the exogenous gene in the patient. In accordance with the instant invention, the compound(s) of the invention is encapsulated or dispersed within the biocompatible, preferably biodegradable polymeric matrix disclosed in PCT/US/03307. The polymeric matrix preferably is in the form of a microparticle such as a microsphere (wherein the compound is dispersed throughout a solid polymeric matrix) or a microcapsule (wherein the compound is stored in the core of a polymeric shell). Other forms of the polymeric matrix for containing the compounds of the invention include films, coatings, gels, implants, and stents. The size and composition of the polymeric matrix device is selected to result in favorable release kinetics in the tissue into which the matrix device is implanted. The size of the polymeric matrix device further is selected according to the method of delivery which is to be used. The polymeric matrix composition can be selected to have both favorable degradation rates and also to be formed of a material which is bioadhesive, to further increase the effectiveness of transfer when the devise is administered to a vascular surface. The matrix composition also can be selected not to degrade, but rather, to release by diffusion over an extended period of time.

Both non-biodegradable and biodegradable polymeric matrices can be used to deliver agents of the invention of the invention to the subject. Biodegradable matrices are preferred. Such polymers may be natural or synthetic polymers. Synthetic polymers are preferred. The polymer is selected based on the period of time over which release is desired, generally in the order of a few hours to a year or longer. Typically, release over a period ranging from between a few hours and three to twelve months is most desirable. The polymer optionally is in the form of a hydrogel that can absorb up to about 90% of its weight in water and further, optionally is cross-linked with multi-valent ions or other polymers.

In general, the agents of the invention are delivered using the bioerodible implant by way of diffusion, or more preferably, by degradation of the polymeric matrix. Exemplary synthetic polymers which can be used to form the biodegradable delivery system include: polyamides, polycarbonates, polyalkylenes, polyalkylene glycols, polyalkylene oxides, polyalkylene terepthalates, polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes and co-polymers thereof, alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, polymers of acrylic and methacrylic esters, methyl cellulose, ethyl cellulose,

hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellulose triacetate, cellulose sulphate sodium salt, poly(methyl methacrylate), poly(ethyl methacrylate), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl alcohols), polyvinyl acetate, poly vinyl chloride, polystyrene and polyvinylpyrrolidone.

Examples of non-biodegradable polymers include ethylene vinyl acetate, poly(meth)acrylic acid, polyamides, copolymers and mixtures thereof.

Examples of biodegradable polymers include synthetic polymers such as polymers of lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, polyurethanes, poly(butic acid), poly(valeric acid), and poly(lactide-cocaprolactone), and natural polymers such as alginate and other polysaccharides including dextran and cellulose, collagen, chemical derivatives thereof (substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications routinely made by those skilled in the art), albumin and other hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof. In general, these materials degrade either by enzymatic hydrolysis or exposure to water *in vivo*, by surface or bulk erosion.

Bioadhesive polymers of particular interest include bioerodible hydrogels described by H. S. Sawhney, C. P. Pathak and J. A. Hubell in Macromolecules, 1993, 26, 581-587, the teachings of which are incorporated herein by reference, polyhyaluronic acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, poly(methyl methacrylates), poly(ethyl methacrylates), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), and poly(octadecyl acrylate).

Use of a long-term sustained release implant may be particularly suitable for treatment of established neurological disorder conditions as well as subjects at risk of developing a neurological disorder. "Long-term" release, as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the active ingredient for at least 7 days, and preferably 30-60 days. The implant may be positioned at or near the site of

the neurological damage or the area of the brain or nervous system affected by or involved in the neurological disorder. Long-term sustained release implants are well known to those of ordinary skill in the art and include some of the release systems described above.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

The preparations of the invention are administered in effective amounts. An effective amount is that amount of a pharmaceutical preparation that alone, or together with further doses, stimulates the desired response. In the case of preventing or treating a disorder or condition that results in excitotoxicity, the desired response is reducing the onset, stage or progression of the excitotoxic damage. This may involve only slowing the progression of the damage temporarily, although more preferably, it involves halting the progression of the damage permanently. An effective amount for preventing and/or treating the excitotoxic damage is that amount that reduces the amount or level of excitotoxic damage and/or increases glutamate transport activity, when the cell or subject is exposed to the neuronal disorder or trauma, with respect to that amount that would occur in the absence of the active compound. The methods of the invention also include methods to prevent or treat glutamate excitotoxicity.

In other embodiments of the invention, (e.g. for making animal models), an effective amount of the pharmaceutical compound is that amount effective to enhance excitotoxic damage and/or inhibit the level of glutamate transport activity. Such enhancements can be determined using standard assays as described above herein. Measurements of glutamate transport activity and/or measurements of excitotoxic damage, are known to those of ordinary skill in the art and may vary depending on the specific neuronal disorder or type of trauma.

The pharmaceutical compound dosage may be adjusted by the individual physician or veterinarian, particularly in the event of any complication. A therapeutically effective

amount typically varies from 0.01 mg/kg to about 1000 mg/kg, preferably from about 0.1 mg/kg to about 200 mg/kg, and most preferably from about 0.2 mg/kg to about 20 mg/kg, in one or more dose administrations daily, for one or more days.

The absolute amount will depend upon a variety of factors, including the material selected for administration, whether the administration is in single or multiple doses, and individual subject parameters including age, physical condition, size, weight, and the stage of the disease or disorder. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation.

Diagnostic tests known to those of ordinary skill in the art may be used to assess the level of glutamate transport activity and/or the level of excitotoxic damage in a subject and to evaluate a therapeutically effective amount of a pharmaceutical compound administered. Examples of diagnostic tests are set forth below. A first determination of glutamate transport activity and/or the level of excitotoxic damage may be obtained using one of the methods described herein (or other methods known in the art), and a subsequent determination of the level of activity and/or damage may be done. A comparison of the activity and/or damage levels may be used to assess the effectiveness of administration of a pharmaceutical compound of the invention as a prophylactic or a treatment of the neurological disorder or trauma. Family history or prior occurrence of a neurological disorder or trauma, even if absent at present, may be an indication for prophylactic intervention by administering a pharmaceutical compound described herein to prevent excitotoxic damage.

An example of a method of diagnosis of excitotoxic damage involves assessing the levels of neuronal cell death, which can be performed using standard methods such as, but not limited to: imaging methods, electrophysiological methods, and histological methods. Additional methods of diagnosis and assessment of chronic and acute neurological disorders and/or trauma and the resulting excitoxicity are known to those of skill in the art.

In addition to the diagnostic tests described above, clinical features of excitotoxicity can be monitored for assessment of glutamate transport levels following neurological disorder or trauma. These features include, but are not limited to: assessment of the presence of neuronal cell lesions, spinal cord lesions, brain lesions, and behavioral abnormalities. Such assessment can be with methods known to one of ordinary skill in the art, such as behavioral testing, CSF evaluation, and imaging studies, such as radiologic studies, CT scans, PET scans, etc.

The pharmaceutical compounds of the invention may be administered alone, in combination with each other, and/or in combination with other drug therapies that are administered to subjects with neurological disorders or trauma. Additional drug therapies (for treatment and/or prophylaxis) that may be administered with pharmaceutical compounds of the invention include, but are not limited to: trophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), glial cell line-derived neurotrophic factor (GDNF), or ciliary neurotrophic factor (CNTF). Other growth factors that may be delivered to the brain and spinal cord include: neurotrophin 4/5 (NT4/5), leukemia inhibitory factor (LIF), cardiotrophin (CT-1), insulin-like growth factors 1 and 2 (IGF-1, IGF-2), transforming growth factor alpha (TGF-alpha), transforming growth factor beta 1-3 (TGF-beta1, TGF beta2, TGF-beta3), neurturin (NTN), artemin (ART), persephin (PSP), acidic fibroblast growth factor (FGF-1), basic fibroblast growth factor (FGF-2), fibroblast growth factor-5 (FGF-5), platelet-derived growth factor (PDGF) and stem cell factor (SCF); amyloid degrading enzymes for Alzheimer's Disease (e.g., the neprilysin (NEP) family of zinc metalloproteinases, such as NEP and endothelin-converting enzyme, insulysin, angiotensin-converting enzyme, matrix metalloproteinases, plasmin and thimet oligopeptidase (endopeptidase-24.15)); glutamate degrading enzymes; anti-oxidants including SOD1, SOD2, glutathione peroxidase and, catalase; anti-apoptotics including Bcl-2, CrmA, baculoviral IAPs and mammalian IAPs (inhibitor of apoptosis proteins including naip, xiap/hilp/miha,c-iap1/hiap-2/mihb, c-iap2/hiap-1/mihc); proteosome enhancers; kinase inhibitors; glutamate transport enhancers (e.g., EAAT2/ GLT1); glutamate metabolizers (e.g., glutamate decarboxylase); beta-amyloid protein antibodies; neurotransmitter synthesizing enzymes including GAD, choline acetyl-transferase and tyrosine hydroxylase; compounds that inhibit caspase activity including caspase inhibitors (e.g., Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk); Z-VDVAD-fmk, Z-DEVD-fmk, and Z-Asp-cmk (Z-Asp-2,6-dichlorobenzoyl-oxymethylketone)), minocycline and dominant negative caspase mutants; haloperidol; phenothiazines; benzodiazepines; acetylcholine esterase inhibitors (including donepezil, rivastigmine and galantamine); tetrahydroacridinamine (Tacrine); beta- and gamma-secretase inhibitors; Abeta vaccines; Cu-Zn chelators; cholesterol-lowering drugs; non-steroidal anti-inflammatory drugs; carbidopa and/or levodopa with or without a catechol-O-methyl transferase (COMT) inhibitors such as Comtan or Tasmar; dopamine agonists including pramipexole, pergolide, and ropinerol; amantadine; selegiline; gabapentin; lamotrigine; topiramate; vigabatrin; Rilutek® (riluzole);

Neurontin® (gabapentin); antispasticity agents including baclofen and tizanidine; cholinergic agents including pyridostigmine; beta blockers including timolol, levobunolol and betaxolol; parasympathomimetics including pilocarbine, carbachol and phospholine iodide; alpha agonists including apraclonidine, brimonidine and epinephrine; carbonic anhydrase inhibitors including dorzolamide and latanoprost; and antistroke agents including antiplatelet agents, anticoagulation agents, thrombolytic agents including plasminogen activators, antithrombotics including anagrelide hydrochloride, bivalirudin, dalteparin sodium, danaparoid sodium, dazoxiben hydrochloride, efegatran sulfate, enoxaparin sodium, ifetroban, ifetroban sodium, tinzaparin sodium and trifenagrel, neuroprotective agents including dizocilpine maleate, platelet activating factor antagonists including lexipafant, platelet aggregation inhibitors including acadesine, beraprost, beraprost sodium, ciprostene calcium, itazigrel, lifarizine and oxagrelate, post-stroke and post-head trauma treatments, cerebral ischemia agents, basic fibroblast growth factors and steroids.

The above-described drug therapies are known to those of ordinary skill in the art and are administered by modes known to those of skill in the art. The drug therapies are administered in amounts that are effective to achieve the physiological goals (to reduce symptoms and damage from neurological disorders and/or trauma in a subject, e.g. excitotoxicity and cell death), in combination with the pharmaceutical compounds of the invention. Thus, it is contemplated that the drug therapies may be administered in amounts which are not capable of preventing or reducing the physiological consequences of the neurological disorder or trauma when the drug therapies are administered alone, but which are capable of preventing or reducing the physiological consequences of neurological disorder or trauma when administered in combination with the glutamate transport modulating compounds of the invention.

According to a further aspect of the invention, pharmaceutical compositions containing the glutamate transport modulators or analogs, derivatives or variants thereof, of the invention are provided. The pharmaceutical compositions contain any of the therapeutic compounds described herein, e.g. the glutamate transport modulators, in a pharmaceutically acceptable carrier. Thus, in a related aspect, the invention provides a method for forming a medicament that involves placing a therapeutically effective amount of the therapeutic compound in the pharmaceutically acceptable carrier to form one or more doses.

The invention also provides a pharmaceutical kit comprising one or more containers comprising one or more of the glutamate transport modulating compounds of the invention

and/or formulations of the invention. The kit may also include instructions for the use of the one or more glutamate transport modulating compounds or formulations of the invention for the treatment of neurological disorder and/or trauma. The kits of the invention, may also comprise additional drugs for preventing and/or treating a neurological disorder.

In another aspect of the invention, cell models and/or non-human animal models of glutamate transport enhancement and/or excitoxicity may be produced by administering an inhibitor of glutamate transport to an animal or contacting a cell with the inhibitor of glutamate transport. Such models may be useful for testing treatment strategies, monitoring clinical features of disease, or as tools to assess prevention strategies of excitotoxic damage in neuronal disorders or trauma. In some embodiments, inhibiting glutamate transport activity is increases glutamate excitotoxicity and a compound that inhibits glutamate transport activity is a compound that increases glutamate excitotoxicity. This increase in glutamate toxicity may be an increase above a control level of glutamate excitotoxicity, but may not be one that results in cell death or other characteristics indicative of a neurological disorder in a subject.

Cells and animal models made using inhibitory compounds of the invention may also be useful for assessing the ability of lead compounds to enhance glutamate transport activity. For example, a cell contacted with an inhibitor of glutamate transport activity may be further contacted with putative agents that are candidate or lead compounds for treating or preventing excitotoxic damage. The ability of the lead or candidate compound to prevent or treat the excitotoxic damage may be evaluated in the model cell or animal. In addition the inhibitors may serve as lead compounds in that if their targets (by definition functionally important) can be identified and characterized, it may subsequently be possible to rationally design new compounds that act as enhancers of these targets.

The invention also relates in some aspects to the identification and testing of candidate glutamate transport modulating compounds. The glutamate transport modulating compounds of the invention can be screened for modulating (enhancing or inhibiting) glutamate transport using the same type of assays as described herein (e.g., in the Example section). Using such assays, the glutamate transport modulating compounds that have the best inhibitory activity can be identified. It is understood that any mechanism of action described herein for the glutamate transport modulating compounds is not intended to be limiting, and the scope of the invention is not bound by any such mechanistic descriptions provided herein.

The invention further provides efficient methods of identifying pharmacological agents or lead compounds for agents and compounds that modulate glutamate transport. Generally, the screening methods involve assaying for compounds which modulate (enhance or inhibit) the level of glutamate transport. As will be understood by one of ordinary skill in the art, the screening methods may measure the level of glutamate transport directly, e.g., screening methods utilizing detectably labeled glutamate, as described above. In addition, screening methods may be utilized that measure a secondary effect of glutamate transport, for example the level of excitotoxicity and/or neuronal cell death in a cell or tissue sample.

A wide variety of assays for pharmacological agents can be used in accordance with this aspect of the invention, including, glutamate transport assays, cell viability assays, cell-based assays, etc. As used herein, the term "pharmacological agent" means glutamate transport modulatory agent. An example of such an assay that is useful to test candidate glutamate transport modulatory agents is provided in the Examples section. In such assays, the assay mixture comprises a candidate pharmacological agent. Typically, a plurality of assay mixtures is run in parallel with different agent concentrations to obtain a different response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration of agent or at a concentration of agent below the limits of assay detection.

Candidate compounds useful in accordance with the invention encompass numerous chemical classes, although typically they are organic compounds. Preferably, the candidate pharmacological agents are small organic compounds, i.e., those having a molecular weight of more than 50 yet less than about 2500, preferably less than about 1000 and, more preferably, less than about 500. Candidate compounds comprise functional chemical groups necessary for structural interactions with proteins and/or nucleic acid molecules. The candidate compounds can comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more of the above-identified functional groups. Candidate compounds also can be biomolecules such as peptides, saccharides, fatty acids, sterols, isoprenoids, purines, pyrimidines, derivatives or structural analogs of the above, or combinations thereof and the like. Where the compound is a nucleic acid molecule, the agent typically is a DNA or RNA molecule, although modified nucleic acid molecules are also contemplated.

It is contemplated that cell-based assays as described herein can be performed using cell samples and/or cultured cells. Biopsy cells and tissues as well as cell lines grown in culture are useful in the methods of the invention.

Candidate compounds are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, synthetic organic combinatorial libraries, phage display libraries of random peptides, and the like. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds can be readily be modified through conventional chemical, physical, and biochemical means. Further, known pharmacological compounds may be subjected to directed or random chemical modifications such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs of the compounds.

A variety of other reagents also can be included in the mixture. These include reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal binding, or to reduce non-specific or background interactions of the reaction components. Other reagents that improve the efficiency of the assay such as protease inhibitors, nuclease inhibitors, antimicrobial agents, and the like may also be used.

An exemplary glutamate transport modulating assay is described herein, which may be used to identify candidate compounds that modulate glutamate transport. In general, the mixture of the foregoing assay materials is incubated under conditions whereby, but for the presence of the candidate pharmacological compound, there is a below-normal level of glutamate transport, although in some embodiments the candidate compound may be one that decreases the level of glutamate transport. The order of addition of components, incubation temperature, time of incubation, and other parameters of the assay may be readily determined. Such experimentation merely involves optimization of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4°C and 40°C. Incubation times preferably are minimized to facilitate rapid, high throughput screening, and typically are between 0.1 and 10 hours.

After incubation, the level of glutamate transport may be detected by any convenient method available to the user. Detection may be effected in any convenient way for cell-based assays. For cell-based assays, one of the components usually comprises, or is coupled to, a

detectable label. For example, glutamate may be detectably labeled. A wide variety of labels can be used, such as those that provide direct detection (e.g., radioactivity, luminescence, optical or electron density, etc.) or indirect detection (e.g., epitope tag such as the FLAG epitope, enzyme tag such as horse-radish peroxidase, etc.).

A variety of methods may be used to detect the label, depending on the nature of the label and other assay components. Labels may be directly detected through optical or electron density, radioactive emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, strepavidin-biotin conjugates, etc. Methods for detecting the labels are well known in the art.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

Examples.

Example 1

Background

We developed a cell-based assay to screen for molecules capable of increasing glutamate uptake activity. Our screening platform consisted of a clonal neural hybrid cell line (referred to here as MN-1) that displayed most of the motor neuron characteristics, including high levels of choline acetyltransferase (CHAT) enzyme activity (Salazar-Grueso et al., *Neuroreport* 2:505-508, 1991), and also showed consistent endogenous glutamate transport activity (Figs. 1, 2). Glutamate uptake in MN-1 cells is mainly accomplished by three Na^+-dependent, high-affinity glutamate transporter subtypes, GLT1, GLAST and EAAC1 as confirmed by western blot (Fig.1), pharmacological and transport kinetics analysis (Fig. 2). MN-1 cells exhibited temperature and Na^+-dependent, high-affinity glutamate uptake linear up to 30 minutes. Experiments using selective inhibitors indicated that the transporter GLT1, which is the main glutamate transporter subtype impaired in ALS, accounted for ~ 40% of the total transport activity in MN-1 cells (Fig. 2B).

As member of the "*Neurodegeneration Drug Screening Consortium*", our laboratory was sponsored by the National Institute of Neurological Disorders and Stroke (NINDS) and the ALS association (Heemskerk et al., *Trends in Neurosciences* 25:494-496, 2002) to screen a library of 1,040 biologically active compounds (MicroSource Discovery Systems, NINDS

custom collection™), looking for glutamate uptake enhancers to be used as potential drugs for therapeutic treatment of amyotrophic lateral sclerosis (ALS), and other neurodegenerative diseases characterized by an impairment of glutamate transporter activity. The collection of compounds was enriched for drugs known to cross the blood-brain barrier, and included FDA-approved drugs, controlled substances, and natural products. The screening protocol includes pre-incubation of the cells for 12 hours with the compounds applied at 1 μM final concentration (in 0.1% DMSO) and the measurement of glutamate uptake activity using ^{3}H-glutamate as radiotracer.

Methods

1) Screening assay.

Embryonic mouse spinal cord motor neuron (MN-1) cells were grown in 24-well plates. When the cells reach near confluence (~90%), the compounds to be screened were added overnight at 1 μM in 0.1% dimethyl sulfoxide (DMSO, final concentration) at 37 °C in Opti-MEM (serum free, Invitrogen, Carlsbad, CA). The control groups received 0.1% DMSO. The next day the compounds were removed by washing 3 times with PBS and the glutamate uptake was measured for 15 min by adding the uptake buffer in the presence of 1 μM ^{3}H-L-glutamate (isotopically diluted). Uptake was then stopped by washing the cells with cold stopping buffer. The cells were harvested using lysis buffer containing 0.5 N NaOH and 0.1% Triton X-100 and placed in scintillation vials, added with Scintisafe® 50% (3ml, Fisher Scientific Co, Pittsburgh, PA.) and counted for radioactivity using a Beckman scintillation counter (Beckman Coulter Inc., Fullerton, CA.)

2) Data Analysis

Uptake values in each well were determined as disintegrations per minute (d.p.m.), which were then converted into pmoles of glutamate taken up in 15 minutes of assay time. Uptake values in drug-treated cells were compared to control vehicle treated (DMSO 0.1%) cells on the same multi-well plate and expressed as percentage of increase or decrease in uptake relative to control.

Standard deviation was within 10% of the uptake values. To define positive hits, we set a cut off that is 3 times the standard deviation. Therefore, 30% increase in glutamate uptake relative to control was indicative of a positive hit. In each 24-well plate we had 3 wells for control and 21 wells for 7 different drugs (7 drugs done in triplicate). There were

control groups in each plate to control for inter-plate variability in uptake values due to conditions of the cells and/or other factors. The compounds selected for EC_{50} analysis were tested at maximum concentration of 30 μM. EC_{50} values are calculated using Grafit software (Erithacus Software, Surrey, UK).

3) Uptake buffer composition

5mM HEPES/Na, 145mM NaCl, 2.5mM KCl, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 1.2mM K_2HPO_4, 10mM Glucose, pH 7.4 in the presence of 1 μM glutamate (isotopic dilution 1:30).

4) Uptake stopping buffer composition

5mM Tris-HCl, 145mM CholineCl, 2.5mM KCl, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 1.2mM K_2HPO_4, 10mM glucose, pH 7.4.

Results

Figure 3 shows representative results obtained by screening 80 compounds from plate #13 of the NINDS collection of compounds. At screening completion, we had identified 8 drugs that significantly increased glutamate uptake in MN-1 (Table 2), with increase ranging from ~30% to ~100% compared to control vehicle treated cells. Preliminary dose-response analysis indicated that these compounds had EC_{50} in the μMolar range (Table 2). Four of these molecules (NDGA, ebselen, flunisolide, and hydrocortisone) are used as anti-inflammatory drugs and their mechanism of action is to interfere with the metabolism of arachidonic acid, a neuromodulator and precursor of pro-inflammatory molecules (Brash, A.R. *J Clin Invest* 107:1339-1345, 2001); NDGA and ebselen also affect oxygen free radical homeostasis by acting as antioxidants (Parnham et al., *Int J Tissue React* 9:45-50, 1987; Schwarz et al., *Carcinogenesis* 5:1663-1670, 1984). These findings are in line with evidence that the activity of the glutamate transporters GLT1, GLAST and EAAC1 is inhibited by poly-unsaturated fatty acids (PUFA) such as arachidonic acid and its breakdown metabolites (Trotti et al., *J Biol Chem* 270:9890-9895, 1995; Volterra et al., *J Neurochem* 59:600-606, 1992), and by reactive oxygen species (Trotti et al., *Trends Pharmacol Sci* 19:328-334, 1998). The other four compounds (iodoquinol, oxyquinolin, cloxyquin and citrinin) have diverse antimicrobial applications, including antibacterial, antibiotic, antiamebic and antiseptic effects.

Table 2. Positive hits from screening of the NINDS library of FDA-approved compounds.

Compound (1 μM)	Therapeutic category	Common Tradenames	% Glutamate uptake increase	EC_{50} (μM)
Nordihydroguaiaretic acid (NDGA)	Lipoxygenases inhibitor, Antioxidant, Anti-inflammatory, Antineoplastic	Masoprocol	108 ± 4.9	1.7
Ebselen	Neuroprotective, Antioxidant, Lipoxygenases inhibitor	N/A	100.5 ± 3	0.5
Flunisolide	Corticosteroid, Anti-inflammatory, antiasthma	Aerobid, Nasalide	38.4 ± 0.5	0.5
Hydrocortisone	Corticosteroid, Anti-inflammatory	Cortef, Hytone	31.7 ± 3.6	0.1
Cloxyquin	Antibacterial	N/A	68.2 ± 13.2	0.7
Citrinin	Antibiotic	N/A	32.5 ± 3.4	0.8
Iodoquinol	Antiamebic	Yodoxin	57.5 ± 6.6	0.3
Oxyquinoline	Antiseptic	Compound present in different multi-ingredient preparations	45.7 ± 4.8	1.7

Example 2

Secondary screen to measure glutamate uptake in synaptosomes from control mice after chronic treatment with the effective drugs.

Background

The following experiments were performed to determine whether restoring the impaired glutamate uptake activity in ALS will have beneficial effects on the progression and/or the onset of the disease. We pharmacologically increased glutamate transport activity in SOD1-G93A ALS mice (see Sutherland, et al. *Soc. Neurosci. Abs* 27: 607.6, 2001) and monitored the progression of the disease. The first step was the validation of the positive hits from the primary screening assay (see Example 1). Then, synaptosomes were used to measure glutamate uptake activity to determine whether chronic treatment of control mice with the glutamate activity modulating compounds increased glutamate transport activity in the central nervous system.

Experimental approach:

1) Drug delivery regimen.

Twenty mice (strain B6SJL-TgNSOD1, control for the SOD1-G93A mice model of ALS) were used to validate the compound. Nordihydroguaiaretic acid (NDGA) was determined to be an effective compound in the primary screening (see Table 1) and its effectiveness was validated in a four-arm study (5 mice/arm). The NDGA compound was delivered initially at three different doses and a placebo was used as control. The drugs were administered to approximately eight-week-old mice at 5, 25, and 50 mg/Kg b.w./day. These dosages correspond to about one tenth of the LD_{50} (dose that is lethal to 50% of the animals) for the compound, as defined by the FDA toxicology reports. The drugs were delivered for 10, 30, and 60 days. Signs of apparent toxicity such as loss of body weight or decreased growth rate, decrease in food intake, inflammatory rectal lesions and hemorrhage were monitored on a daily basis. In case of toxicity at the lowest dose in the study, the dosage would be lowered, initially by a factor of two.

2) Route of drug delivery.

The active compounds and placebo were delivered in a time-controlled release manner using subcutaneous implantation of matrix-driven delivery pellets (Innovative Research of America; Sarasota, FL). This technology integrates the principles of drug diffusion, erosion and concentration gradient by generating a finished pellet with a biodegradable matrix that effectively and continuously releases the active product in the animal in a time-controlled manner. Placebo pellets were used in experiments as the proper control to interpret results obtained with pellets containing active products. Matching placebo pellets contained all the components of the drug pellets except the active product itself. The carrier-binder excipients of the pellet matrix included cholesterol, lactose, cellulose, phosphates and stearates. The pellets were implanted using a 10 gauge precision trochar on mice that are 8 weeks old and weigh approximately 20g. Pellets that were tailored for 10 days delivery were used. Therefore, for the 60-day treatment, we implanted a total of 6 pellets.

3) Glutamate uptake measurement in mice.

Glutamate uptake was measured using synaptosomes prepared from the forebrain of mice treated. Synaptosomes are intact vesicular structures derived by homogenization and

centrifugation (Gray and Whittaker, *J. Anat.* 96: 79-88, 1962) and formed by plasma membrane fragments of neurons and astrocytes which forms chemical synapses in the central and peripheral nervous system. This preparation exhibits glutamate transport activity that reflects the function of glutamate transporters expressed in astrocytes and neurons at the excitatory synapses (Volterra, et al., *J Neurochem* 59(2):600-6, 1992). Compounds that consistently increase glutamate uptake in a time- and dose-dependent manner, as measured in synaptosomes, are considered as candidates for therapeutic treatment of ALS mice. The effect on glutamate uptake monitored on synaptosomes also validated the compound's ability to cross the blood-brain-barrier and become bio-available in the nervous system.

Methods

1) Pellet implantation procedure.

The mice were anesthetized with isoflurane. The pellet was then placed in the trochar and the skin on the lateral side on the neck of the animal was lifted and the trochar inserted. When the pellet in the trochar was contacts the skin, the trochar was twisted sideways and the pellet inserted. The total distance from the incision was ~2 cm. If the technique was properly executed no antibiotics or stitches were needed and the animal did not bleed and did not experience any pain or distress.

2) Synaptosomes preparation and uptake measurement.

Synaptosomes were prepared from mice forebrains according to the method of Gray and Whittaker (Gray and Whittaker, *J. Anat.* 96: 79-88, 1962) with small modifications (Volterra et al., *J Neurochem* 59(2):600-6, 1992). Synaptosomes (50 µg/ml) were incubated at 37 °C for 15 min under shaking conditions with oxygenated Krebs/bicarbonate buffer containing: 124 mM NaCl, 4.6mM KCl, 1.2mM $CaCl_2$, 1.3mM $MgCl_2$ x $6H_2O$, 0.42mM KH_2PO_4, 26.75mM $NaHCO_3$, 10mM glucose, adjusted to pH 7.4 and 10 µM [^{3}H]-glutamate (N.E.N. 41.9 Ci/mmol; isotopic dilution 1:4,000). The uptake was terminated by addition of ice cold buffer with 100-fold excess cold glutamate and rapid filtration on 0.45 µm nitrocellulose filters. Filter blotted synaptosomes were counted by liquid scintillography for incorporated radioactivity.

3) Properties of the glutamate uptake assay.

Accumulation of glutamate in synaptosomes in the presence of Na^+ was usually between 100,000 and 140,000 decays per minute (d.p.m.) at the isotopic dilution indicated above. In the absence of Na^+ the accumulation was generally less than 5% of the total accumulation of radiolabel. The intra-assay variability was quite low; triplicate measures were within 5%. The data were calculated as follows:

$$\text{Uptake velocity} = \frac{(\text{d.p.m. in presence of } Na^+ - \text{d.p.m. in absence of } Na^+)}{2.22 \times 10^{12} \text{ d.p.m./Ci x specific activity of Glu in Ci/mol x 15 min x mg protein}}$$

Results

In vivo testing of the glutamate transport enhancer Nordihydroguaiaretic acid (NDGA)

Nordihydroguaiaretic acid (NDGA) was tested in mammals *in vivo* as described. The compound was delivered by subcutaneous implantation of small pellets as described, and the results confirmed the effectiveness of the secondary screening. NDGA and placebo pellets (10 days) were given to control mice at 50mg/Kg body weight/day. After 10 days, the mice were sacrificed and glutamate uptake measured in forebrain synaptosomes as described. The results, represented in Fig. 5A, show the effect of NDGA (10mg/10 days) on glutamate uptake measured in forebrain synaptosomes of control mice, which indicates that the NDGA treatment increased the glutamate uptake activity by ~50% compared to placebo. Fig. 5B shows glutamate uptake in synaptosomes in control mice (single values/mouse).

Example 3

Cloxyquin, citrinin, iodoquinol, oxyquinoline, ebselen, flunisolide, and hydrocortisone are tested using the methods described in Example 2 to determine their effectiveness in modulating glutamate uptake activity *in vivo*. In addition, analogs, derivative, and variants of cloxyquin, citrinin, iodoquinol, oxyquinoline, ebselen, flunisolide, hydrocortisone, and NDGA are tested to determine their effectiveness in modulating glutamate uptake activity *in vivo*.

Example 4

SOD1-G93A mice, a model for amyotrophic lateral sclerosis (ALS), were treated with NDGA. The experiment was designed to rescue the animals from ALS-like symptoms and/or to alleviate the ALS-like symptoms in the animals by administering drugs that are effective in

increasing glutamate uptake in the brain. NDGA was administered via pellet implantation under the skin in the back of the neck of these mice, as described in Example 2. The pellets were a cellulose matrix embedded with NDGA and designed to release the drug into the bloodstream in a time-dependent manner. The dose utilized was 10mg of NDGA for 10 days of continuous release, which resulted in a 1mg/day dosage.

The NDGA regimen is represented in Fig. 6. Insertion of the NDGA-containing pellet or placebo was done in mice at 100 days of age, which is the age at which the mice develop the first symptoms of ALS. The experiment was designed to treat the mice at disease onset and thereby provide a correlate for a therapeutic treatment approach in humans.

The effectiveness of NDGA was evaluated by looking at the survival curve. Placebo or untreated mice all died at 145 days of age because of the progression of the ALS-like symptoms. The NDGA-treated mice all died by 155 days of age, demonstrating a right shift in the survival curve. On average the mean survival for the placebo (control) group and the NDGA group did not differ: 133.6 ± 2 days versus 135.5 ± 2.4. (Fig. 7) However, the results indicated a trend of extended survival in a subgroup of animals. This suggested that a greater impact on survival may be obtained with administration of a higher dosage of NDGA.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

All references, including patent documents, disclosed herein are incorporated by reference in their entirety.

We claim: